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14. ABSTRACT During this second year of the project, we established multiple primary cell strains from normal controls and SSc patients. We performed stimulation assays with silica in 82 primary fibroblast strains. Our results showed that silica activate fibroblasts toward fibrotic changes. However, different fibroblast strains obtained from different individuals showed different responses in terms of the gene expression of the ECM components. Using longitudinal linear models in analysis of association between specific genotypes and dynamic changes of gene expression of the fibroblasts in responding to silica stimulation, we identified that specific SNPs were associated with either single gene expression, or paired gene expression such as CTGF/SPARC and COL1A2/COL3A1, which suggest a strong biological correlation between these genes. Moreover, some SNPs and/or their corresponding genes were found to be associated with both SSc susceptibility and the fibrotic changes of human fibroblast in response to silica stimulation. These SSc susceptibility genes include not only previously identified ones, but also some novel ones, such as HLA-DPB1 and APBA1. These observations supported our original proposal that genetic elements within SSc fibroblasts might contribute to susceptibility to fibrotic process. Integrative studies of genetic and environmental factors with human fibroblasts may facilitate the discovery of potential pathogenesis of SSc. In this year, we will continuously obtain more human fibroblasts, especially SSc fibroblasts. We also will continuously perform stimulation assays of newly obtained human fibroblasts for a better chance to identify genetic components inside SSc patients contributing to susceptibility to environmental stimuli. Meanwhile, we will try to explore which specific bio-pathways associated with which specific genetic factors involved in silica induced fibrotic changes. Therefore, our studies are fulfilled with original proposal in the grant.					
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Introduction:

This project aims to study interactions between genetic and environmental factors in a viable system - human fibroblasts. Fibroblasts obtained from scleroderma (SSc) patients have a profibrotic nature that suggests a possibility of a dysregulation of biological function in the cells. On the other hand, SSc occurs in genetically susceptible individuals. A SSc susceptible genetic background may be more vulnerable to environmental triggers. Studies of biological functions of fibroblasts with and without SSc susceptible backgrounds in response to potential environmental triggers will provide a great opportunity to understand etiopathogenesis of SSc.

Body:

According to our SOW, there are four major tasks that will be performed for this project. 1. We will culture and genotype human fibroblasts obtained from skin biopsies. 2. We will stimulate cultured fibroblasts with silica individually, and then examine potential alterations of fibroblasts in terms of proliferation, collagen synthesis, cytokine expression and release. In addition, stimulations with cellulose, titanium oxide and carbon particles will be used as negative controls. 3. We will apply comprehensive statistical analysis to identify the association of genetic susceptibility genes and these environmental factors in terms of the degree of their profibrotic effects. 4. We will also study specific biological pathways triggered by risk elements in cultured fibroblasts.

During this third year of the project, we have been continuously obtaining skin biopsies from SSc patients and normal controls. For a new update, we have a total of 188 skin fibroblast strains including 74 from SSc patients and 114 from normal controls. We will have 12 more enrollments to reach a total of 200 fibroblast strains proposed in the grant. We are very optimistic about new enrollment. Although, it appeared that we have more controls enrolled in the project, if these controls have potential SSc genotypes, they would compensate some patient enrollment. However, we still expect 26 new patients enrolled this year. These primary fibroblast cultures were established from each skin biopsy, and maintained in healthy condition for proposed functional studies.

We have been performing the HLA genotyping on these new patients and controls. It should be noted that we recently replaced our traditional genotyping methods (proposed in the grant) with sequencing method for all classic HLA genes using the SeCore™ Kits of Invitrogen (Carlsbad, California). This new HLA typing method is much more efficient compared to the traditional one. The SeCore strategy begins with the amplification of the target locus by cycling an amplification mix, FastStart™ Taq DNA Polymerase and sample genomic DNA (PCR). The resulting product is treated with ExoSAP-IT™, prior to sequencing, to degrade the unincorporated primers and hydrolyze the free nucleotides. The nucleotide chronology, and resulting HLA subtype is determined by multicolor, fluorescence-based, DYEnamic ET Terminator sequencing. The final reactions are purified by an ethanol precipitation prior to loading. Denatured samples are loaded and results detected on an automated sequencing ABI3730. Sequencing data will be read using a HLA sequencing software Conexio Genomics Assign 3.5.

We have performed stimulation assays on 104 primary fibroblast strains for part of our proposed studies. Each of these 104 fibroblast strain was stimulated with silica particles for evaluation of their responses toward potentially fibrotic changes over 5 different time-points. The fibroblast strains with increased collagen gene expression (COL1A2 and COL3A1) upon stimulation are further examined for collagen content, proliferation and cytokine expression (IL1A, IL6, TNFA and ICAM-1).

For data analysis, Dr. Momiao Xiong (co-investigator) developed both single and multi-level multivariate longitudinal linear genetic models for investigation of associations between genetics and time-course gene expressions of fibroblasts in response to silica stimulation. Specifically, in the single longitudinal linear model, the expression level of a single gene is taken as a quantitative trait, while it is taken from the expression levels of three genes for multi-level multivariate longitudinal linear model. Specific genotype of a gene that are tested for influencing gene expression is referred to as eQTL. Time-course gene expressions of the fibroblasts in association with genetic susceptibility and environmental (such as silica particles) perturbations are a function of time t . We view the time-course

gene expressions as a realization of a stochastic process and decompose them into mean function, and genetic additive effect and dominance effect functions using Fisher's factor analysis and variation method, which leads to a general genetic model of functional trait and formulates genetic effect functions as function of genotypic values and allele frequencies. We applied these two models in 79 fibroblast strains that were assayed for multiple gene expressions in response to a time-course silica stimulation, and also were genotyped with the genome-wide single nucleotide polymorphism (SNP) arrays (Illumina Human 371 K SNP panel). For quantitative trait, we used the expression data of single collagen genes (COL1A2 or COL3A1) in single longitudinal linear model, and three extracellular matrix genes (COL3A1, CTGF and SPARC) in multivariate longitudinal linear model. For eQTL, we used genome-wide SNP types of each individual's fibroblasts. Single and multivariate longitudinal linear models each identified multiple genotypes (SNPs) associated with the expressions of collagen genes and three ECM genes, respectively. The most striking result is that both models identified that the SNP rs10484710 was strongly associated with the single gene expressions of COL1A2 and COL3A1, and with the combination expressions of COL3A1, CTGF and SPARC (Table 1). Rs10484710 is an intronic SNP of collagen type XXI, alpha 1 gene. It also was shown in association with SSc susceptibility ($p = 6.96 \times 10^{-4}$) according to the genome-wide association studies performed by Dr. Maureen Mayes, a professor in our division of Rheumatology in UT Health Science Center (Dr. Mayes' personal communication). Currently, we are preparing a manuscript based on the results reported here and some from last year.

Table 1. Associations between SNP rs10484710 and the expressions of the ECM genes
Single longitudinal Linear model

gene expression	p value for regulating expression	OR
COL1A2	5.9×10^{-13}	1.4
COL3A1	1.65×10^{-5}	1.4

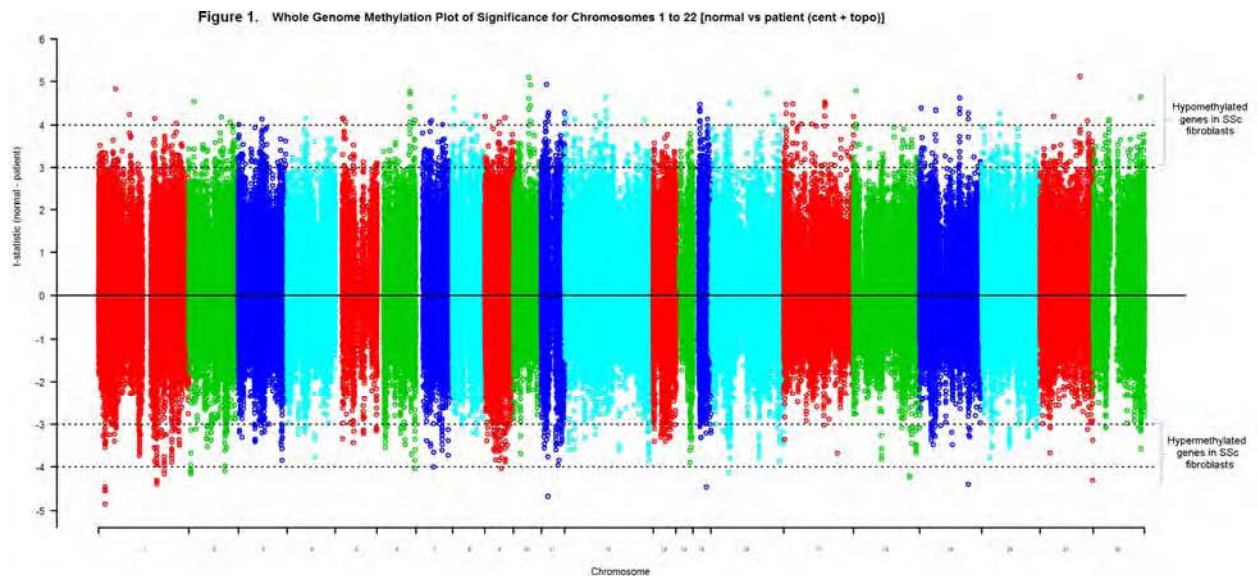
Multi-level multivariate longitudinal linear model		
gene expression	p value for regulating expression	OR
COL3A1, CTGF, SPARC	5.11×10^{-8}	1.4

In the studies of bio-pathways associated with SSc pathogenesis, we continued to explore attenuation of fibrosis through TGF- β pathway which can be induced by silica particles according to our previously reports. We applied gene specific siRNAs of SPARC and/or CTGF to attenuate fibrotic changes in the fibroblasts obtained from TGF- β transgenic mice. We also treated with these siRNAs in fibrotic mouse model induced by bleomycin that is considered as another environmental trigger for scleroderma. The paper has been recently published in the Arthritis Research & Therapy (PMID: 20359365). In addition to these two fibrotic models, we also examined anti-fibrotic effects of SPARC siRNA and CTGF siRNA in the CTGF transgenic model since the CTGF is a down-stream gene in TGF- β pathway. Our results showed that inhibition of Sparc or Ctgf expression by their corresponding siRNA in cultured fibroblasts of *Col1a2-CTGF* transgenic mice down-regulated the expression of TGF β and collagen type I. Sparc and Ctgf siRNAs also showed a reciprocal inhibition at transcript levels, but Sparc siRNA functioned more efficiently than Ctgf siRNA in reducing the protein level of both Sparc and Ctgf (see attached manuscript). It should be noted that although, the *in vivo* studies of anti-fibrosis were funded by other projects, the results represent a successful example of translational studies of medical diseases.

Silica exposure has been linked to anti-nuclear autoantibodies (ANA) and other autoantibodies in SSc (*Haustein UF, Ziegler V et al. J Am Acad Dermatol. 1990;22:444-8*). Anti-DNA topoisomerase I (topo I) antibodies were reported to be the predominant autoantibodies present in silica-associated SSc (*McHugh NJ, Whyte J, Arthritis Rheum. 1994;37:1198-205*). Topo I is an important nuclear protein that catalyzes the breaking and joining of DNA strands and controls DNA replication and transcription. We recently examined whether and how the catalytic function of topo I is changed, and its potential association with fibrosis in SSc fibroblasts. Our studies indicated that

topo I molecules were altered in their function with relocation in the nucleus. In some fibroblasts, especially those obtained from skin biopsies of SSc patients who were positive for anti-topo I or anti-RNA polymerase III autoantibodies, these alterations were associated with increased sumoylation of topo I, which may facilitate relocation of topo I molecules. In contrast, the fibroblasts of anti-centromere positive patients showed unchanged sumoylation of topo I. Inhibition of SUMO1 gene improved catalytic function of topo I and down-regulated collagen gene expression in SSc fibroblasts. These observations may provide important insights into the nature of SSc fibroblasts that may contribute to pathological processes, induction of an autoimmune response to topo I, and/or disease development in SSc (see attached manuscript).

It is worth to note that we recently received an exploratory research fund from our institution to study DNA methylation of SSc fibroblasts. DNA methylation is regarded as one of the most significant epigenetic events, which can be induced by various environmental factors. It is heritable and plays important roles in disease pathogenesis. We just completed a pilot study of DNA methylation profiling of fibroblasts from 16 SSc cases and 10 controls using Roche NimbleGen 385K RefSeq Promotor arrays. SSc fibroblasts showed a global gene hypomethylation (Figure 1), especially in anti-topo I autoantibodies positive patients. Examples of important hypomethylated genes include IL6R, SMAD6, CADH11 and COL4A1. It may be interesting to study whether silica exposure can induce any similar changes in DNA methylation of human fibroblasts.



Overall, our data strongly support our original proposal that genetic elements within SSc fibroblasts might contribute to susceptibility to fibrotic process. Integrative studies of genetic and environmental factors with human fibroblasts may facilitate the discovery of potential pathogenesis of SSc.

Key Research Accomplishments

- Obtained a total of 188 human fibroblast strains (74 SSc patients and 114 normal controls).
- Completed silica stimulation in 104 fibroblast strains and obtained RNA and protein extracts from each of the experiments.
- Up-grade our HLA genotyping method with sequencing techniques and auto-reading program (software Conexio Genomics Assign 3.5).
- Completed genotyping for 79 fibroblast strains (34 SSc and 45 controls) with genome-wide SNP profiling, and HLA genotyping for 110 patients.
- Identified that SNP rs10484710 of the COL21A1 is strongly associated with gene expression changes of collagens and other ECM elements involved in activation of fibrosis.

- Demonstrated that specific inhibition of SPARC and/or CTGF attenuated mouse fibrosis induced by bleomycin (an environmental factor for SSc) *in vivo*, as well as inhibited fibrotic changes in fibroblasts obtained from both TGF- β and CTGF transgenic mice.
- Identified that catalytic function of topoisomerase I (topo I) was decreased in SSc fibroblasts, which appeared to be associated with increased sumoylation of topo I. Inhibition of sumoylation of topo I improved the topo I function, and down-regulated collagen I gene expression in SSc fibroblasts.
- Preliminary studies showed that DNA methylation of SSc fibroblasts was significantly changed compared to normal control fibroblasts, which suggests that environmental factors may contribute to SSc through epigenetic changes.

Reportable Outcomes

During this third year period, we published three papers, presented two abstracts and completed two manuscripts (see the list below).

We established a total of 188 human fibroblast strains (74 SSc and 114 controls), which is close to our minimum target of 200 that can be used for many studies toward human health.

Our postdoctoral fellow, Dr. Jiucun Wang (PhD), completed her training and accepted a faculty position in Fudan University, Shanghai, China. Currently, Dr. Khurshida Begum is taking over the postdoctoral fellow position supported by this grant. Dr. Begum was graduated with a PhD from Okayama University in Japan. Before she moved to this position, she was a postdoctoral fellow in the Molecular Genetics & Biotechnology Laboratory in the Texas A&M University.

Referred articles (underline for corresponding author):

1. Wang JC, Lai SL, Guo XJ, Zheng XF, de Crombrughe B, Arnett FC and **Zhou X D.** Attenuation of fibrosis in vitro and in vivo with Sparc siRNA. *Arthritis Research & Therapy*, 2010 Apr 1;12(2):R60. **PMID: 20359365**
2. Momiao Xiong, Gang Peng, Li Luo, Yun Zhu, Pengfei Hu, Shengjun Hong, Jinying Zhao, **Zhou XD**, John Reveille, Li Jin, Christopher Amos. Gene and Pathway-Based Analysis - Second Wave of Genome-wide Association Studies. *European Journal of Human Genetics*. 2009, 8. **PMID:19584899.**
3. **Zhou XD**, Lee JU, Arnett FC, Xiong MM, Park MY, Yoo YK, Shin ES, Reveille JD, Mayes MD, Kim JH, Song R, Choi JY, Park JA, Lee YJ, Lee EU, Song YW, Lee EB. HLA-DPB1 and DPB2 are culprit genetic loci for susceptibility to systemic sclerosis, Genome-wide association study in Koreans with Replication in North Americans. *Arthritis Rheum*. 2009;30;60(12):3807-3814. **PMID: 19950302**

Abstract (see attachment):

1. Association studies of genetic variants to the functions of human fibroblasts in response to environmental stimulation. Jiu-Cun Wang, Xinjian Guo, Liming Li, Wensheng Guo, Momiao Xiong, Filemon.k.Tan, Frank C. Arnett, and Xiaodong Zhou
2. Single and multi-level multivariate longitudinal models in the genetic studies of time course gene expressions of human fibroblasts in response to environmental stimulation. Momiao Xiong, Li Luo, Yu-li Lin, Frank Alert, Wenyaw Chan and Xiaodong Zhou

Manuscript submitted (see attachment):

1. **Zhou, XD.** Lin, W., Tan, F.K., Guo, XJ. and Arnett, F.C. Altered Sumoylation and Function of DNA Topoisomerase I in Scleroderma Fibroblasts
2. Wang JC, Guo XJ, Zhang XF, Arnett FC, de Crombrughe B, **Zhou XD**. Attenuation of gene expression of extracellular matrix elements with siRNAs of Sparc and Ctgf in cultured skin fibroblasts from Ctgf transgenic mice.

1. **Zhou XD**, Jagannath C, Tan FK, Guo XJ, Tejpal N, Kahan BD, Stepkowski SN, Arnett FC. Up-take of silica and carbon nanotubes by human macrophages induces activation of T cells and fibroblasts in vitro – potential implication for pathogenesis of fibrotic diseases.
2. JC Wang, Xiong H, Tan FK, Arnett FC, **Zhou XD**. Studies of genetic and environmental factors in human fibroblasts showed novel gene-gene interactions.

Conclusion

During this third year of the project, we established multiple primary cell strains from normal controls and SSc patients. We performed stimulation assays with silica in 104 primary fibroblast strains. Our results showed that different fibroblast strains obtained from different individuals responded differently to silica stimulation in terms of the gene expression of the ECM components that are involved in activation of fibrosis. Using both single and multi-level multivariate longitudinal linear models in analysis of association between specific genotypes and dynamic changes of gene expression of the fibroblasts in responding to silica stimulation, we identified multiple genotypes (SNPs) associated with the expressions of collagen genes and three ECM genes. The most striking result is that both models identified that the SNP rs10484710 of COL21A1 was strongly associated with the single gene expressions of COL1A2 and COL3A1, and with the combination expressions of COL3A1, CTGF and SPARC. This SNP also was associated with SSc susceptibility ($p = 6.96 \times 10^{-4}$) according to a recent genetic study of SSc in over 1600 Caucasian SSc patients in our Division. These observations supported our original proposal that genetic elements within SSc fibroblasts might contribute to susceptibility to fibrotic process. Integrative studies of genetic and environmental factors with human fibroblasts may facilitate the discovery of potential pathogenesis of SSc. Recently, we are obtaining HLA genotyping data from an up-graded sequencing method. Once all samples are genotyped, we will perform analysis with comprehensive biostatistic methods. In addition to above main stream studies, we also perform some functional studies of SSc fibroblasts aiming to finding bi-pathways associated with fibrosis. We identified that silencing SPARC and/or CTGF attenuated fibrotic changes *in vivo* and *in vitro* induced by TGF- β signaling (can be induced by silica) and/or bleomycin (another environmental trigger for SSc). We also identified that catalytic function of topo I was decreased in SSc fibroblasts, and which appeared to be associated with increased sumoylation of the topo I. Inhibition of SUMO expression improved the topo I function and down-regulated COL1A2 gene expression in SSc fibroblasts. These novel observations provided us a potential mechanism underlying dysfunction of SSc fibroblasts.

In this year, we will continuously obtain more human fibroblasts, especially SSc fibroblasts. We also will continuously perform stimulation assays of newly obtained human fibroblasts for a better chance to identify genetic components inside SSc patients contributing to susceptibility to environmental stimuli. Meanwhile, we also will explore potential mechanisms such as specific bio-pathways underlying the changes of SSc fibroblasts. Therefore, our studies are fulfilled with original proposal in the grant.

Appendices (two manuscripts and two abstracts submitted for publication)

manuscript 1

Decreased Catalytic Function with Altered Sumoylation of DNA Topoisomerase I in Scleroderma Fibroblasts

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Abstract

Objective: The presence of autoantibodies to nuclear and nucleolar proteins is a unique and prominent feature in systemic sclerosis (SSc) and suggests a possibility of functional alteration of the nuclear proteins. On the other hand, profibrotic nature of SSc fibroblasts indicates that a dysregulation of biological function occurs in the cells. Topo I is an important nuclear protein that controls DNA replication and transcription. The aims of this study were to examine the catalytic function of topo I and its associated sumoylation in SSc fibroblasts.

Methods: Twelve pairs of fibroblast strains were obtained from upper-arm skin biopsies of SSc patients and age/sex/ethnicity matched normal controls. Catalytic function of topo I was evaluated using the nuclear extracts of fibroblasts or recombinant human topo I to relax supercoiled DNA. Immunoprecipitation (IP) Western blots were used to examine topo I sumoylation in fibroblasts. SUMO1 siRNA was used to inhibit SUMO1 expression, while enzymatic SUMO1 reactions were used to increase sumoylation of topo I. In addition, real-time quantitative RT-PCR was used for measuring transcript levels of SUMO1 and collagen type I in the fibroblasts.

Results: Topo I in nuclear extracts of SSc fibroblasts generally showed a significantly lower efficiency than that of normal fibroblasts in relaxing the same amount of supercoiled DNA. Increased sumoylation of topo I evaluated by IP Western blots was clearly observed in 8 of 12 SSc fibroblast strains, in which 3 each from anti-topo I and anti-RNA polymerase III positive patients, and 1 each from patients with anti-fibrillarin autoantibodies or unknown antibody status. Four SSc fibroblast strains including two each from patients with anti-centromere and non-SSc specific autoantibodies, showed similar levels of sumoylation to their normal counterparts. Inhibition of SUMO1 with SUMO1 siRNA improved the catalytic efficiency of topo I in nuclear extracts of the SSc fibroblasts, and which was accompanied by a mild reduction of transcript levels of the COL1A2. In contrast, sumoylation of recombinant topo I proteins reduced their catalytic function.

Conclusion: Catalytic function of topo I was decreased in SSc fibroblasts, to which increased sumoylation of topo I may contribute at least in part. Inhibition of SUMO1 improved topo I function as well as attenuated profibrotic collagen expression in cultured SSc fibroblasts.

Introduction

SSc is a human multi-system fibrotic disease with high morbidity and mortality. Etiology and pathogenesis of SSc are still unknown.

Fibrosis in skin is the most common clinical presentation in SSc patients. Based on the extent of skin involvement, SSc is classified into limited cutaneous and diffuse cutaneous forms. The latter subset is characterized by more rapid progression of skin and visceral involvement, as well as poorer prognosis (1,2). Fibrotic skin is characterized by excessive deposition of collagens and other extracellular matrix (ECM) components produced in connective tissues. Skin fibroblasts obtained from SSc patients have been found to be profibrotic to synthesize large amounts of ECM proteins, which contributes to tissue fibrosis (3). It is believed that a possible defect in regulation of biological functions is present in SSc fibroblasts.

Majority of SSc patients (95%) have autoantibodies against various nuclear and nucleolar proteins, which include non-specific antinuclear antibodies (ANA) and a group of SSc-specific antibodies. Anti-DNA topoisomerase I (topo I) autoantibody is SSc-specific, and it occurs in 20-25% of patients (4,11,12). There is a strong correlation between anti-topo I autoantibody and the diffuse cutaneous form of SSc (11,12). Levels of anti-topo I autoantibody have been reported to correlate with disease severity and activity in SSc and the lack of these antibodies conveys a better outcome in SSc (13). In addition to anti-topo I, there are several other SSc specific autoantibodies. The most common ones are directed against centromeric proteins (ACA) that are associated with limited cutaneous fibrosis, RNA polymerases (I, II and III) (ARA) that are associated with diffuse skin fibrosis, and fibrillarin that is associated with diffuse skin thickening and visceral involvement (14). Topo I is a monomeric 100 kD nuclear protein. It catalyzes the breaking and joining of DNA strands prior to transcription (6,7), and is associated with nuclear functions involving transcription,

DNA replication, and chromatin condensation. Topo I translocates between the nucleolus and the nucleoplasm, but is enriched in the nucleolus where there is a high level of transcription and replication of the ribosomal DNA (6,7). Sumoylation is a post-translational modification, in which the substrates covalently attach the small ubiquitin-like modifier (SUMO) to their lysine residues. Sumoylation of topo I has been shown to facilitate its movement between the nucleolus and the nucleoplasm (8-10).

The purpose of this study was to determine whether there is abnormal function, distribution and/or sumoylation of topo I in fibroblasts obtained from SSc patients.

Materials and Methods

Dermal fibroblast cultures

Skin biopsies (3 mm punch biopsies) were obtained from the upper arms of 12 pairs of age and gender matched SSc patients and normal controls. All SSc patients fulfilled American College of Rheumatology criteria for SSc (15). All patients had disease duration of less than five years. Normal controls were identified with no history of autoimmune diseases undergoing dermatologic surgery. All subjects provided informed consent and the study was approved by the Committee for the Protection of Human Subjects at The University of Texas Health Science Center at Houston.

Each skin sample was transported in Dulbecco's Modified Essential Media (DMEM) with 10% fetal calf serum (FCS) (supplemented with an antibiotic and antimycotic) for processing the same day. The tissue sample was washed in 70% ethanol, PBS, and DMEM with 10% FCS. Cultured fibroblast cell strains were established by mincing tissues and placing them into 60 mm culture dishes secured by glass coverslips. The primary cultures were maintained in DMEM with 10% FCS and supplemented with antibiotic and antimycotic. The early passage (< 5 passage) fibroblast strains were plated at a density of 2.5×10^5 cells in a 35 mm dish and grown for assays accordingly.

Catalytic function of topo I in SSc fibroblasts

Nuclear proteins were extracted from equal amount of fibroblast cells obtained from cultures by using nuclear extract kits (Active Motif, Carlsbad, CA). The Topoisomerase I Assay kit (TopoGEN Inc, Port Orange, FL) was used for measuring catalytic function of topo I. Briefly, supercoiled DNA substrate (0.25 μ g) (TopoGen) was reacted with nuclear proteins containing topo I at serial dilutions. After 30 minute incubation at 37°C, the reaction was terminated with stop buffer (5% Sarkosyl, 0.125% bromophenol blue and 25% glycerol). The reaction mixtures were loaded on a 1 % agarose gel, and then stained with ethidium bromide. The catalytic activity of topo I was determined by measuring intensity of the supercoiled DNA bands after reactions with a serial dilution of topo I in the nuclear extract of fibroblasts. A Bio-imaging system (Gene Genius, Syngene, Frederick, MD) was used to scan the bands in agarose gel. The Gene Snap software (Syngene) was used to quantify the intensity of the bands. A total of 12 pairs of SSc and control fibroblast strains were examined with this assay.

Immunostaining

SSc and normal fibroblasts were grown in culture media as described above. After 7, 14 and 18 days, the cells were washed with PBS and fixed with 100% methanol at 4°C for 2 minutes. The cells were washed with PBS again, and incubated with serum from SSc patients (evenly pooled from 4 SSc patients) who had positive anti-topo I autoantibodies, or monoclonal antibodies of mouse anti-human topo I or mouse anti-human SUMO 1. This was followed by incubation with secondary antibodies with fluorescence (rabbit anti-human IgG antibodies and anti-mouse

antibodies). DAPI was used as nucleus counterstain. The images of fibroblasts with fluorescence labeled proteins were acquired using fluorescence microscopy (Nikon Eclipse TE2000-4).

Western blotting

Nuclear extracts from cultured fibroblasts were measured for protein concentration by a spectrophotometer. Equal amounts of protein from each sample were subjected to SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred onto nitrocellulose membranes and incubated with 1:1000 diluted primary antibodies including mouse anti-human topo I (ImmunoVision, Springdale, AR), or anti-human SUMO1 (ABGENT, San Diego, CA) individually. The secondary antibody was a peroxidase-conjugated anti-mouse IgG. Specific proteins were detected by chemiluminescence using an ECL system (Amersham, Piscataway, NJ). The intensity of the bands was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation (IP) Western blotting

The total number of about 3.5×10^7 fibroblast cells of each subject were harvested with trypsin and washed twice with 25ml ice-cold PBS/phosphatase inhibitors. Cell pellets were gently resuspended by 2ml hypotonic buffer. Nuclear extracts were isolated and measured for protein concentration by a spectrophotometer. Equal amounts of protein (500 ug) from each sample were subjected to immunoprecipitation (IP) with mouse anti-SUMO-1(GMP1, Invitrogen, Carlsbad, CA) and then were subjected to SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred onto nitrocellulose membranes and incubated with 1:1000 diluted primary antibodies of mouse anti-human topo I (ImmunoVision). The secondary antibody was a horseradish peroxidase-conjugated anti-mouse IgG (eBioscience, San Diego, CA). Specific proteins were detected by chemiluminescence using supersignal west pico stable peroxide solution (Thermo Scientific, Rockford, IL). The intensity of the bands was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Inhibition of SUMO1 with siRNA transfection in fibroblasts

SUMO1 siRNAs were purchased from Invitrogen. Three SSc fibroblast strains that showed stronger sumoylation of topo I and weaker catalytic topo I function were used for transfection of SUMO1 siRNA. Briefly, the fibroblasts were grown at a density of 1.5×10^5 cells in 25-cm² flasks until confluency. The culture medium (DMEM) in each culture flask was replaced with Opti_MEM 1 (Invitrogen) without FCS. The fibroblasts were transfected with SUMO siRNA using Lipofectamine RNAiMAX (Invitrogen) in a concentration of 15 ug/ml. A fluorescein-labeled non-silencing control siRNA (Qiagen, Valencia, CA) was used for detection of transfection efficiency. After 24 hours, the culture medium was replaced with normal DMEM. The fibroblasts were examined for gene and protein expression, as well as topo I catalytic function after 48- or 72-hour transfection.

Sumoylation assay of topo I

Recombinant human topo I was purchased from TopoGEN Inc. A mixture containing topo I protein, SUMO-1 protein (Active Motif, Carlsbad, CA), activating enzyme E1/conjugating enzyme E2 (Active Motif) and sumoylation buffer (15 mM ATP, 25 mM MgCl₂ and 250 mM Tris-HCl) was incubated at 30 °C for 3 hours. A mutant SUMO-1 protein (Active Motif) lacking of sumoylation function was used as a negative control. Reaction was stopped with 5 mM EDTA. The recombinant

human topo I with and without sumoylation were examined with Western blotting and topo I catalytic assays. The experiments were performed in triplicates.

Quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR) for measurement of SUMO1 expression, as well as COL1A2 expression after SUMO1 siRNA transfection

The primers and probes of SUMO1, COL1A2, 18S and GAPDH were obtained from the Assays-on-Demand product line from Applied Biosystems (Foster City, CA). Total RNA from each sample was extracted from the cultured fibroblasts described above using a total RNA kit from OMEGA Biotek (Norcross, GA) after treatment with DNase I. Complementary DNA (cDNA) was synthesized using SuperScript II reverse transcriptase (Invitrogen). Synthesized cDNAs were mixed with primer/probe of SUMO1 or COL1A2 in 2 x TaqMan universal PCR buffer and then assayed on an ABI Prism 7900 Sequence Detector System (Applied Biosystems). Each sample was assayed in triplicates. The data were analyzed with SDS2.2 (ABI). The amount of each transcript was normalized with 18S and GAPDH levels.

Measurement of autoantibodies

Patient's sera were tested for antinuclear antibodies by indirect immunofluorescence using HEp-2 cells as antigen substrate (Antibodies Inc, Davis, CA). Anti-topo I antibodies were detected by passive immunodiffusion against calf thymus extracts (INOVA Diagnostics, Ingbert, Germany), anti-RNA polymerase III antibodies were detected by ELISA using commercial kits (NBL, Nagoya, Japan). Anti-centromere antibodies were determined visually by their distinctive immunofluorescence patterns on HEp-2 cells. Anti-fibrillarin antibodies were detected by immunoprecipitation as described previously.

Results

Reduced catalytic function of topo I in SSc fibroblasts

After catalytic reactions with a serial dilution of topo I in the nuclear extracts, the supercoiled DNA band was gradually diminished following increased amounts of topo I in the nuclear extracts. Based on the intensity of supercoiled DNA bands that were correlated with the amounts of topo I in the nuclear extracts, the efficiency of SSc topo I in relaxing the supercoiled DNA appeared to be less than that of control topo I in each concentration of nuclear extracts (Figure 1A). Overall comparison of catalytic functions of topo I in all 6 dilutions of nuclear extracts between SSc and control fibroblasts was statistically significant ($p = 0.0041$) (Figure 1B).

Altered localization of topo I in SSc fibroblasts

When anti-topo I monoclonal antibodies were used as probes, the majority of SSc fibroblasts from each patient showed strong nucleoplasm staining (multiple speckles) compared to normal fibroblasts in which topo I staining showed normal enrichment in the nucleolus (Figure 2A). A few SSc fibroblasts (less than 1%) showed cytoplasmic staining of topo I molecules which was not observed in normal fibroblasts. However, there were more SSc fibroblasts (approximately 2%) showing cytoplasmic staining of topo I molecules when anti-topo I positive sera from SSc patients were used as probes (Figure 2B). The cytoplasmic staining of topo I appeared to be stronger at 14 or 18 days of culture compared to 7 days of culture. Discrepancy between the findings of topo I staining in SSc fibroblasts with SSc positive sera versus monoclonal antibodies may be due to different epitopes on topo I molecules recognized by the two sources of antibodies.

Altered sumoylation of topo I in SSc fibroblasts

Western blotting showed that quantitative levels of topo I proteins were similar between SSc and normal control fibroblasts, while SUMO 1 levels were increased in SSc fibroblasts. To validate this finding, we examined sumoylated topo I in the nuclear proteins using IP Western blotting (Figure 3). Increased sumoylation of topo I evaluated by IP Western blots was clearly observed in 8 of 12 SSc fibroblast strains including 3 each from anti-topo I and anti-RNA polymerase III positive patients, and 1 each from patients without specific SSc autoantibodies or unavailable serum.

Interestingly, increased poly-sumoylation of topo I was shown in the fibroblast strains of all 3 anti-topo I positive patients, 2 anti-RNA polymerase III positive patients and 1 without specific SSc autoantibodies (Figure 3). Four SSc fibroblast strains including two each from patients with anti-centromere and without SSc autoantibodies, showed similar levels of sumoylation to their normal counterparts.

Inhibition of SUMO1 in SSc fibroblasts increased catalytic function of topo I

A real-time quantitative RT-PCR assay showed that inhibition of SUMO1 with siRNA achieved a significant reduction of gene expression of SUMO1 (Figure 4). Compared to non-target siRNA transfected fibroblasts, SUMO1 siRNA transfected fibroblasts showed a 30.97-times reduction of SUMO1 expression ($p < 0.001$, T test) along with a mild reduction of the COL1A2 ($p = 0.045$). Importantly, compared to either non-target siRNA transfected or non-siRNA transfected fibroblasts, catalytic function of topo I of transfected SSc fibroblasts showed a marked improvement in all three test fibroblast strains (Figure 5).

Sumoylation of recombinant topo I decreased its catalytic function

Recombinant human topo I proteins were sumoylated with either wild type SUMO1 or mutant SUMO1 or negative control (without sumoylation) and then were examined with Western blots for sumoylated topo I and with topo I catalytic assays for topo I function. Poly-sumoylation of topo I was observed in the topo I proteins sumoylated with wild type SUMO1 (Figure 6). Sumoylation of topo I with wild type SUMO1 showed a reduction of efficiency in catalytic function compared to the topo I protein sumoylated with mutant sumo 1 or negative control (Figure 7). The assays were performed in triplicates, which showed similar results.

Discussion

A novel finding of these studies is decreased catalytic function of topo I in human SSc fibroblasts, and which is surprisingly not specific to the patients with anti-topo I autoantibodies. Human topo I plays important roles in DNA metabolic processes, such as transcription and replication, in which it releases topological stress in DNA chains (6,7). Topo I is generally condensed in the nucleolus where a high level of transcription and replication of the ribosomal DNA occur. In response to inhibitory factors to topo I, such as camptothecin, UV irradiation and transcription inhibitors, topo I molecules were usually relocated from the nucleolus to the nucleoplasm with unclear mechanisms. Interestingly, SSc fibroblasts examined herein showed enhanced staining of topo I in the nucleoplasm, which suggests a relocation of topo I, and also support a reduced function of topo I-associated DNA metabolic processes. The cytoplasmic staining of topo I observed in some SSc fibroblasts was mainly detected by anti-topo I positive serum from SSc patients and was inconsistent with that found using anti-topo I monoclonal antibodies. Considering that the human sera may contain mainly unpurified antibodies, both non-specific and antigen specific cross-reactions of antibodies to cytoplasmic proteins are possible. For these potential antigen specific cross-reactions, mitochondrial topo I has high amino acid homology to nuclear topo I. On the other hand, it is also possible that the detected cytoplasmic staining of topo I may represent degraded topo

I molecules processed by proteasomes. It is worth noting that the topo I autoantigenic component, a 70 kD polypeptide, has been reported to be exported via ectocytosis in SSc fibroblasts (16), and anti-topo I autoantibodies of SSc patients were shown to bind to SSc fibroblasts (17).

Sumoylation is an important post-translational modification. Previous studies have indicated that sumoylation of topo I facilitates a translocation of topo I protein from nucleolus to nucleoplasm (8-10). An increased sumoylation of topo I in the majority of SSc fibroblasts observed herein supports a potential mechanism that may drive the movement of topo I from the nucleolar compartments to the nucleoplasm where a degradation process may occur in proteasomes. To further investigate the association between altered sumoylation and topo I function in SSc fibroblasts, we inhibited the SUMO1 expression with sequence specific SUMO1 siRNA. Interestingly, SUMO1 inhibition induced a favorable improvement of catalytic function of topo I in examined fibroblasts, thus suggesting that decreased topo I function observed in SSc fibroblasts may be a result of increased sumoylation. This possibility was consistent with the follow-up studies of sumoylation of recombinant human topo I that showed a reduction of catalytic function. However, sumoylation may not fully explain the reduction of topo I function in all SSc fibroblasts, especially in those fibroblasts which did not show the changes of sumoylation of topo I. These fibroblasts include two each from patients with ACA and with other ANAs. In contrast, the fibroblasts from all 6 patients with either anti-topo I or ARA showed hyper-sumoylation of topo I. It is worth noting that the presence of either anti-topo I or anti-RNA polymerase III autoantibodies in SSc patients is correlated with the diffuse form of SSc and internal organ fibrosis, while the anti-centromere positive patients usually have a limited form of SSc with favorable clinical outcomes. Recent studies of SSc genetics have indicated that different genetic susceptibility markers may determine the types of autoantibodies presenting in SSc patients (18,19). The characteristic patterns and specific genetic associations of SSc autoantibodies suggest that distinctive mechanisms contribute to different autoantibody-associated SSc subsets.

Topo I is essential in biological function of human cells. Previous reports indicated that loss of the topo I gene was associated with death of animals at an early stage of embryogenesis (20,21). Inactivation of the topo I gene *in vitro* was found to induce genomic instability with chromosomal aberrations (22). Inhibition of topo I function through camptothecin or topotecan (a camptothecin derivative) in human HEP-2 cells altered nuclear structure and function and induced recruitment of topo I for proteasomal degradation (23). In fact, topotecan used therapeutically for cancer has been reported to induce SSc-like disease (24). Whether decreased catalytic function of topo I in SSc fibroblasts examined herein may result in any consequences associated with pathological changes in SSc is worthy of further investigations. Interestingly, the recovery of topo I function in SSc fibroblasts after inhibition of SUMO1 was accompanied with a mild reduction of collagen genes including the COL1A2 and the COL3A1 (Figure 4). This observation suggested that collagen expression may be influenced by either the SUMO1 level or topo I function in SSc fibroblasts.

In summary, our studies of topo I in SSc fibroblasts indicate that topo I molecules are altered in their function with relocation in the nucleus. In some fibroblasts, especially those obtained from skin biopsies of SSc patients who were positive for anti-topo I or anti-RNA polymerase III autoantibodies, these alterations were associated with increased sumoylation of topo I. In contrast, the fibroblasts of anti-centromere positive patients showed unchanged sumoylation of topo I. Inhibition of SUMO1 gene improved catalytic function of topo I in SSc fibroblasts. These observations may provide important insights into the nature of SSc fibroblasts that may contribute to pathological processes, induction of an autoimmune response to topo I, and/or disease development in SSc.

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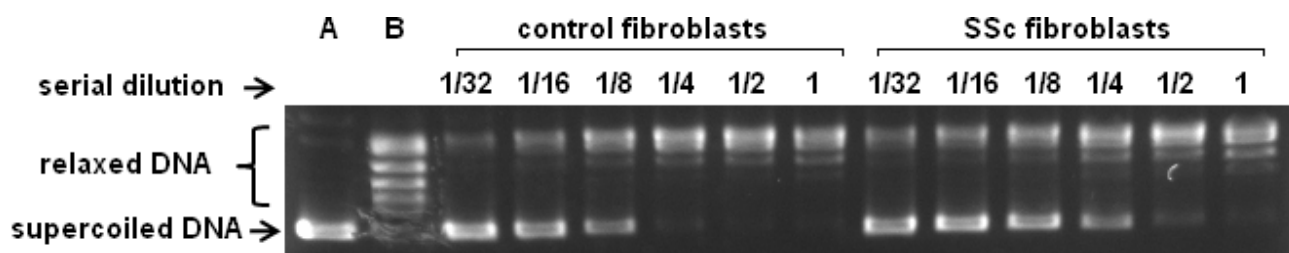
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Figure 1. Measurement of catalytic function of topo I in cultured fibroblasts. A serial dilution of topo I in the nuclear extracts obtained from SSc and control fibroblasts were used to relax 0.25 µg supercoiled DNA.

1A. The supercoiled DNA band is gradually diminished following increased amounts of topo I in the nuclear extracts in the relaxing assays. The efficiency of SSc topo I in relaxing the supercoiled DNA appeared to be less than that of control topo I in each concentration of nuclear extracts.

1b. Comparison of SSc and control fibroblasts for mean values of intensity of supercoiled DNA bands after relaxing assay with different concentrations of topo I in the nuclear extracts. Each *p* value of comparison at different dilution points is listed in the figure. Overall comparison in all 6 dilutions between SSc and control fibroblasts showed a significant *p* value (*p* = 0.0041). A = standard supercoiled DNA band; B = standard relaxed DNA bands; the numbers (1/32, 1/16, 1/8, 1/4, 1/2 and 1) indicate serial dilutions of topo I in nuclear extracts used for relaxing supercoiled DNA.

1B.



1b.

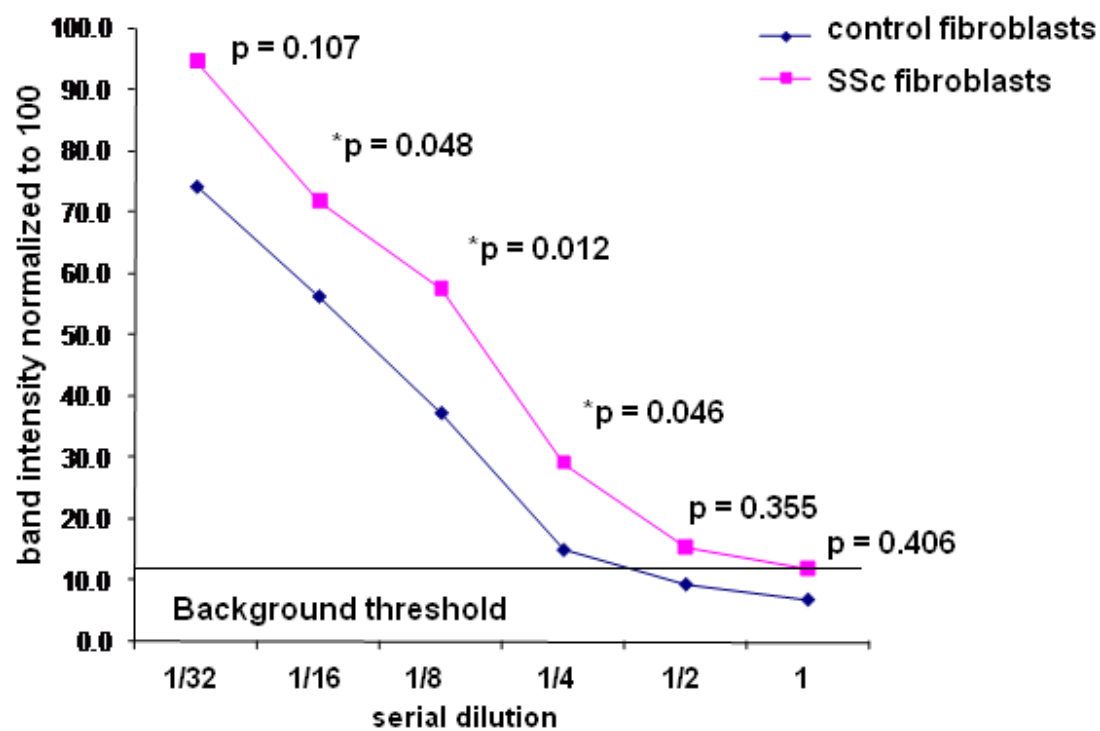
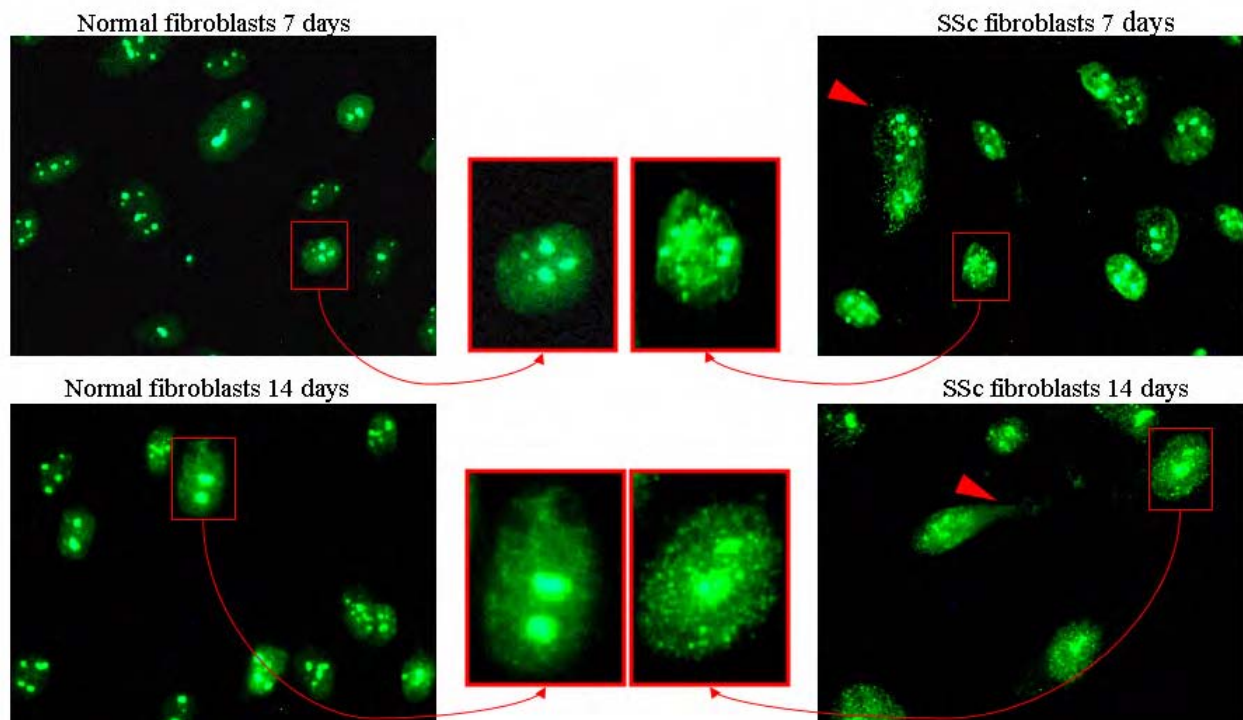


Figure 2. A. Comparison of topo I staining with anti-topo I monoclonal antibodies in cultured fibroblasts of normal controls and SSc patients. Topo I immunostaining with anti-topo I monoclonal antibodies as probe showed multiple speckles in the nucleoplasm in SSc fibroblasts, which is differentiated from that in normal fibroblasts (relatively homogenous stain of topo I) at both 7 and 14 days of cultures. Some SSc fibroblasts show cytoplasmic staining for topo I protein (marked with red arrows). B. Topo I immunostaining with anti-topo I positive sera from SSc patients as probe. Cytoplasmic staining of topo I in SSc fibroblasts appeared to be stronger than that in the nucleoplasm and nucleoli of a SSc fibroblast strain at day 14.

A.



B.

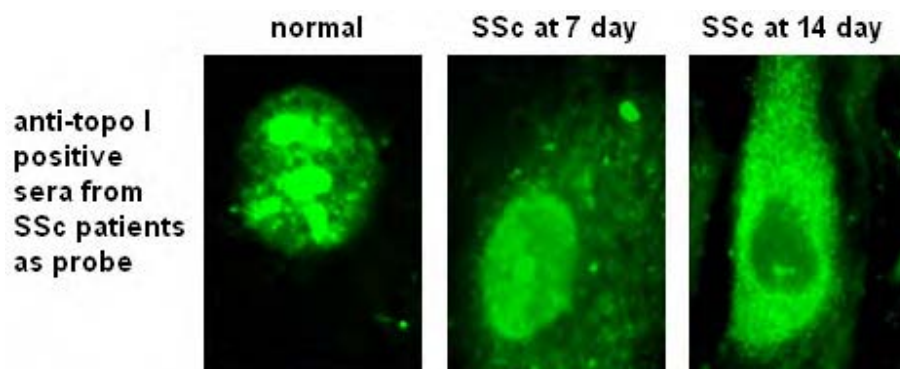


Figure 3. Immuno-precipitated Western blots and autoantibody profiles for 12 SSc patients. Each SSc patient (SSc) has an age and sex matched normal control (C) for comparison of sumoylated topo I expression with IP Western blots. Poly-sumoylated Topo I appeared in SSc fibroblast strain number 1, 2, 3, 4, 8 and 10. ANA: antinuclear antibodies

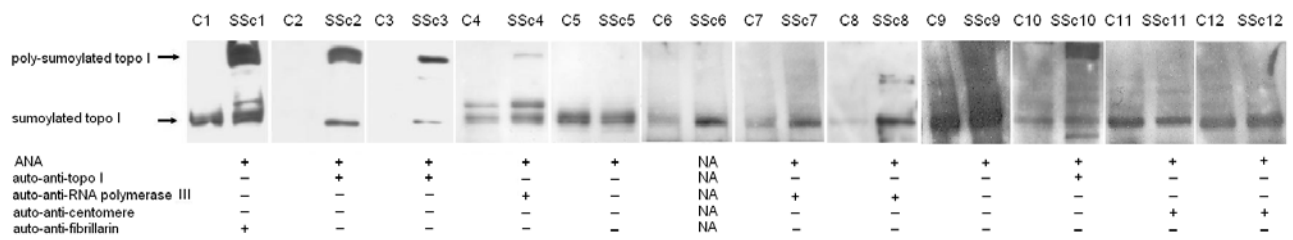


Figure 4. Real time RT-PCR for transcript level of SUMO1 with and without SUMO1 siRNA transfection in SSc fibroblasts. Three SSc fibroblast strains were transfected with SUMO1 siRNA. After 48-hour transfection, total RNAs were used for measuring SUMO1 transcript levels along with COL1A2.

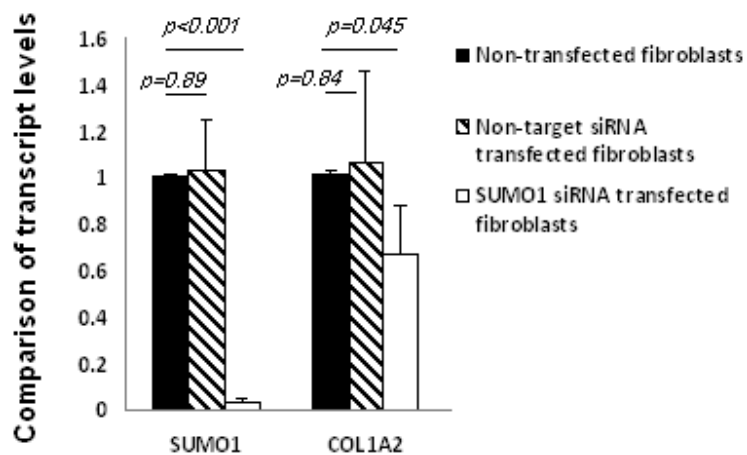


Figure 5. Measurement of catalytic function of topo I in cultured SSc fibroblasts with and without SUMO1 siRNA transfection. A serial dilution of the nuclear extract containing topo I obtained from SSc fibroblasts were used to relax 0.25 µg supercoiled DNA. The supercoiled DNA band is completely turned into relaxed DNA bands between the dilution 1/2 and 1 in the fibroblasts without siRNA transfection or non-target siRNA transfection. In contrast, this change is observed between the dilution 1/8 and 1/4 in the fibroblasts with SUMO1 transfection, which indicates a higher efficiency of catalytic function of topo I after SUMO1 inhibition in the fibroblasts.

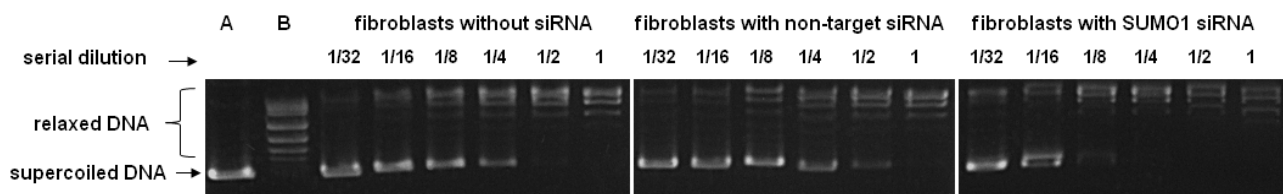


Figure 6. Western blots show sumoylation of recombinant human topo I. Recombinant human topo I protein was subjected to sumoylation reaction, and examined with Western blotting using anti-topo I (I) and anti-SUMO1 antibodies (II). Compared to topo I protein without sumoylation reaction (topo IA), topo I protein with sumoylation reaction (topo IB) showed poly-sumoylation of topo I (II).

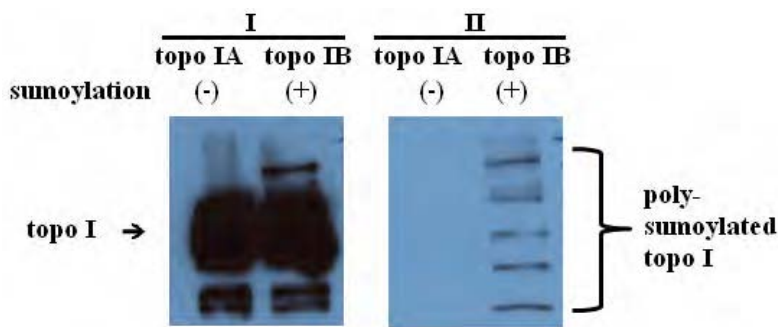
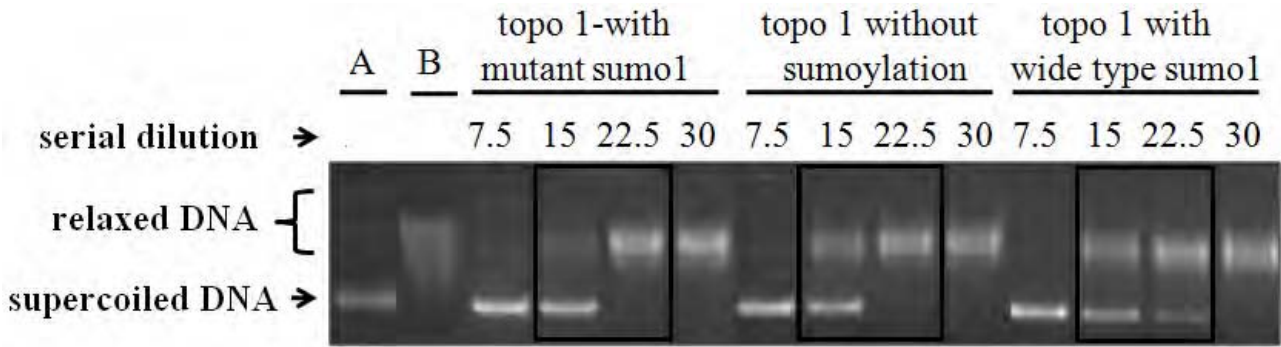


Figure 7. Measurement of catalytic function of recombinant human topo I with and without sumoylation reaction. Recombinant human topo I proteins were sumoylated with either mutant sumo1 or wild type sumo1 or negative control (without sumoylation), and then were examined for their catalytic function in a serial dilution. Sumoylation of topo I with wild type sumo1 showed a reduction of efficiency in catalytic function (supercoiled DNA disappeared at the dilution of topo I concentration of 30) compared to the topo I protein sumoylated with mutant sumo1 or negative control (supercoiled DNA disappeared at topo I concentration of 22.5). *A: standard supercoiled DNA band, B: standard relaxed DNA bands



Manuscript 2

**Attenuation of gene expression of extracellular matrix elements with siRNAs
of Sparc and Ctgf in cultured skin fibroblasts from *Col1a2-CTGF* transgenic mice**

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Objective: *Col1a2-CTGF* transgenic mice that over-express *Ctgf* in fibroblasts with a *Col1* promoter showed a SSc-like fibrotic phenotype. This study is to investigate the regulation of *Sparc* and *Ctgf* siRNAs on the expression of several extracellular matrix components in the fibroblasts derived from the novel ***Col1a2-CTGF* transgenic** mouse model. **Methods:** MEFs from ***Col1a2-CTGF* hemizygous, homozygous and wild-type littermate controls** were transfected with *Sparc* siRNA or *Ctgf* siRNA. Real-time quantitative RT-PCR and Western blotting were used to examine the transcription and/or protein levels of type I collagen, *Ctgf*, *Sparc*, and *Tgfb1*. Student's paired t-tests were used to determine the significance of the results. **Results:** *Col1a2*, *Ctgf*, and *Sparc* showed increased expression at both transcriptional and translational levels in the fibroblasts from the *Col1a2-CTGF* transgenic mice compared with fibroblasts littermate wild-type controls. The transfection with *Sparc* siRNA or *Ctgf* siRNA attenuated the mRNA and/or protein expression of the *Col1a2*, *Ctgf*, *Sparc* and *Tgfb1* in these fibroblasts. The attenuation of *Ctgf* protein level by *Ctgf* siRNA is less efficient than that of *Sparc* protein by *Sparc* siRNA **Conclusion:** Inhibition of *Sparc* or *Ctgf* expression by their corresponding siRNA in cultured fibroblasts of *Col1a2-CTGF* transgenic mice showed a down-regulation of the expression of TGF β and collagen type I. *Sparc* and *Ctgf* siRNAs also showed a reciprocal inhibition at transcript levels, but *Sparc* siRNA functioned more efficiently than *Ctgf* siRNA in reducing the protein level of both *Sparc* and *Ctgf*.

Systemic sclerosis (SSc), also known as scleroderma, is a complex autoimmune disease characterized by skin and internal organ fibrosis. Currently, there is neither effective therapy nor effective prevention for this disease. Although the etiology of SSc is still unknown, both *in vitro* and *in vivo* studies have indicated that the extensive deposition of collagens, and other extracellular matrix (ECM) proteins by activated fibroblasts is a major pathologic property of SSc (1,2).

To better understand the pathogenic mechanisms and to find potential therapeutic targets of SSc, several animal models have been reported, including genetically modified mice harboring disruptions or manipulation of pivotal signaling pathways, have been established (2,3). Transforming growth factor β (TGF β) is a fibrotic growth factor. Overactivity of TGF β signaling has been widely accepted to play important roles in the fibrosis of SSc (4). Connective tissue growth factor (CTGF) is a downstream mediator of TGF β signaling. Many of the profibrotic properties of TGF β might be induced by the actions of CTGF (5). *Col1a2-CTGF* transgenic mice that over-express CTGF in fibroblasts under the control of the fibroblast-specific collagen $\alpha 2(I)$ promoter enhancer showed some aspects of fibrosis, resembling those seen in human Scleroderma. The animal models provide a platform for testing potential anti-fibrotic therapies for SSc.

SPARC (secreted protein, acidic and rich in cysteine) is a matricellular component of the ECM. It participates in the modulation of cell-matrix interactions, cell adhesion, wound repair, and angiogenesis (7-9) and possibly plays an important role in fibrosis. Increased expression of SPARC have been found in many fibrotic diseases including SSc, pulmonary fibrosis, renal interstitial fibrosis, hepatic cirrhosis, and atherosclerotic vascular lesions (10-14). SPARC has been shown to interact with the TGF β signaling pathway possibly through the TGF β receptor and mediated through a Smad2/3-dependent mechanism (15).

In our previous studies, we observed that SPARC can regulate the expression of type 1 collagen, a major structural protein of the ECM, in normal human fibroblasts (16). Moreover, after exogenous TGF β stimulation, SPARC siRNA showed a protective role against overexpression of collagen genes (16). Specific inhibition of SPARC expression with siRNA led to a down-regulation of collagen and CTGF gene expression in SSc fibroblasts (17). In order to evaluate the influence of the inhibition of Sparc in Ctgf transgenic mouse models and its potential as a therapeutic target of SSc, *in vitro* study was performed using fibroblasts derived from *Col1a2-CTGF* transgenic mouse embryonic fibroblasts (MEFs) to investigate the regulation of Sparc siRNA on the expression of several ECM components, and to compare it with that of Ctgf siRNA.

MATERIALS AND METHODS

Cell lines. Fibroblasts were prepared from *Col1a2-CTGF* hemizygous, homozygous and littermate WT MEFs.. The cultures were maintained in DMEM with 10% FCS and supplemented with antibiotics (50 U/ml penicillin and 50 μ g/ml streptomycin). Fifth-passage fibroblast cells were seeded on gelatin coated plates at a density of 5×10^5 cells in 25-cm² flasks and grown until confluence.

Transient transfection with siRNA. Double-stranded ON-TARGET $plus$ siRNAs of murine *Sparc* and *Ctgf* were purchased from DHARMACON (Lafayette, CO). The corresponding target sequences are 5'- GCACCACACGUUUCUUUG -3' for *Sparc* and 5'- GCACCAGUGUGAAGACAUA -3' for *Ctgf*, respectively. The culture medium in each culture flask with confluent fibroblasts was replaced with Opti-MEM I medium (Invitrogen, Carlsbad, CA) without FCS and antibiotics. The fibroblasts were transfected with Sparc siRNA or Ctgf siRNA, using Metafectene (Biontex, Munich, Germany) in a concentration of 3 μ g siRNA per ml medium. Fibroblasts with Non-Targeting siRNA treatment were used as

negative control. After 8 hours, the culture medium was replaced with DMEM. The cells transfected with siRNA were examined after 72 hours of transfection and used for RNA expression and protein assays.

Determination of gene expression by quantitative RT-PCR. Quantitative real-time RT-PCR was performed using an ABI 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). The specific primers and probes for each gene (*Col1a2*, *Ctgf*, *Sparc*, *Tgfb1*) were purchased from the Assays-on-Demand product line (Applied Biosystems). Total RNA from each sample was extracted from the cultured fibroblasts using RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized using MultiScribe™ Reverse Transcriptase (Applied Biosystems). Synthesized cDNAs were mixed with primers/probes in 2 × TaqMan universal PCR buffer and then assayed on an ABI 7900 sequence detector. The data obtained from the assays were analyzed with SDS 2.2 software (Applied Biosystems). The amount of total RNA in each sample was normalized with *Gapdh* transcript levels.

Western blot analysis. The cellular lysates extracted from the above cultured fibroblasts were used for protein assays. The protein concentration was determined by a spectrophotometer using Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein from each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Resolved proteins were transferred onto PVDF membrane and incubated with respective primary antibodies, including anti-type I collagen antibody (Bioscience International, Saco, ME), anti-Ctgf antibody (GeneTex Inc, San Antonio, TX), and anti-Sparc antibody (R&D Systems Inc, Minneapolis, MN). Mouse β -actin (Alexis Biochemicals, San Diego, CA) was used as an internal control. The secondary antibody was peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse IgG. Specific proteins were detected by chemiluminescence using an enhanced

chemiluminescence system (Amersham, Piscataway, NJ). The intensity of the bands was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Col1a2, Ctgf, Sparc and Tgfb1 expression in Ctgf transgenic mice

fibroblasts: As measured by quantitative real-time RT-PCR, *Col1a2*, *Ctgf*, *Sparc* and *Tgfb1* showed increased expression in the fibroblasts from both types of Ctgf transgenic mice compared with those in the cells from their normal littermates (wide type) except the *Tgfb1* expression in homozygotes. (Figure 1). The folds changed of each gene in heterozygous and homozygous fibroblasts were 2.11 ± 0.01 , 1.54 ± 0.23 for *Col1a2*, 5.77 ± 0.36 , 7.09 ± 1.57 for *Ctgf* (, 1.66 ± 0.18 , 1.79 ± 1.05 for *Sparc*, and 1.59 ± 0.19 , 0.86 ± 0.12 for *Tgfb1*, respectively. The expression trends of *Ctgf* in heterozygous and homozygous fibroblasts were in accordance with the transferred Ctgf transgene number in the cells. However, the expression of *Sparc* was similar in the two cell lines and *Col1a2* expression was contrarily higher in heterozygotes than in homozygotes

Transfection efficiency: Two methods were used for measuring transfection efficiency of siRNA. First, the fibroblasts were transfected with fluorescein-labeled nonsilencing siRNA, and then examined under a fluorescence microscopy. It showed ~80% transfection efficiency by direct cell counting. Second, the fibroblasts from GFP transgenic C57BL/6 mouse (The Jackson Laboratory, Bar Harbor, Maine) were transfected with specific siRNA of GFP, and then examined as to how many cells responded with decreased levels of GFP. A similar efficiency of transfection was seen (Figure 2).

Gene expression of *Col1a2*, *Ctgf*, *Sparc* and *Tgfb1* after transfection of siRNAs in wild type and *Col1a2-CTGF* MEFs: Seventy-two hours after Ctgf siRNA or

Sparc siRNA transfection, the reduction of *Ctgf* by *Ctgf* siRNA and *Sparc* by *Sparc* siRNA were observed in all the three fibroblast lines from wild type or *Ctgf* transgenic mice by 73%-87% for *Ctgf* and 68%-82% for *Sparc*, respectively (Figure 3). Furthermore, the knockdown of *Ctgf* by *Ctgf* siRNA was more efficient than that of *Sparc* by *Sparc* siRNA in all three fibroblast lines (73% vs 69%, 85% vs 82%, and 83% vs 68% in wild type, heterozygous and homozygous fibroblasts, respectively). The expression of *Col1a2*, *Ctgf*, *Sparc* and *Tgfb1* were consistently inhibited in all the fibroblasts after transfection with either *Ctgf* siRNA or *Sparc* siRNA by 26% to 87%. The fold changes of down-regulation of *Col1a2* by *Ctgf* siRNA was higher than that by *Sparc* siRNA in these fibroblasts (62% vs 41%, 74% vs 58%, and 70% vs 50% in wild type, heterozygous and homozygous fibroblasts, respectively), while the reciprocal down-regulation of *Sparc* by *Ctgf* siRNA and *Ctgf* by *Sparc* siRNA was similar.

Protein expression of *Col1a2*, *Ctgf* and *Sparc* in the fibroblasts with or without siRNA treatment: The expression of the three ECM components at protein level was examined by Western blot analysis. Collagen type 1, *Ctgf*, and *Sparc* showed increased expression in the fibroblasts of both hemi- and homozygous *Col1a2-CTGF* MEFs compared with those in the cells from their normal littermate controls (Figure 4), which was in accordance with their increased expression at mRNA level. However, the fold increase of each protein in heterozygous and homozygous fibroblasts was less than those of each mRNA. They were 1.26 ± 0.11 , 1.13 ± 0.01 for Col1, 2.73 ± 1.23 , 1.72 ± 0.46 for *Ctgf*, and 1.19 ± 0.18 , 1.32 ± 0.20 for *Sparc*, respectively. The expression trends of Col1 and *Sparc* were similar with those of corresponding mRNA, while the *Ctgf* expression in homozygous fibroblasts was lower than that in heterozygous fibroblasts.

Western blots also showed that the expression of Col1, *Ctgf* and *Sparc* were decreased after *Ctgf* siRNA or *Sparc* siRNA treatment in all the three fibroblast lines except for *Sparc* expression in wild type and heterozygous fibroblasts with *Ctgf* siRNA

treatment (Figure 4). However, the pattern of attenuation of each protein level was different from that of mRNA. The down-regulation of Ctgf protein by Ctgf siRNA was obviously less efficient than that of Sparc protein by Sparc siRNA (27% vs 86%, 20% vs 89%, and 39% vs 92% in wild type, heterozygous and homozygous fibroblasts, respectively).

Furthermore, the down-regulation of Sparc protein by Ctgf siRNA was also less than that of Ctgf protein by Sparc siRNA (-10% vs 16%, 1% vs 32%, and 33% vs 40% in these fibroblasts, respectively). The reduction of Col1a2 protein by Ctgf siRNA was less than that by Sparc siRNA in wild type and heterozygote (37% vs 47%, 21% vs 35%, and 41% vs 41% in these fibroblasts, respectively), but similar in homozygote. The data also revealed that the homozygote appeared to be more sensitive to the treatment of either Sparc siRNA or Ctgf siRNA at protein level than the heterozygote, according to the decreased levels of above ECM proteins (Figure 4), while it was not the case at mRNA level (Table 1 and Figure 3).

DISCUSSION

Scleroderma is a devastating fibrotic disease which confers a high risk of mortality. No optimal therapy for reducing excessive collagen production in this disease is available (17). Animal model studies are crucial step in finding therapeutic targets in SSc (18). Recently, Ctgf transgenic mouse models, including both heterozygotes and homozygotes, were generated, that express a constitutively over-expression of Ctgf (6). These mice displayed features similar to human scleroderma including dermal fibrosis and lung fibrosis and thus provided useful tools in the study of fibrogenesis and identification of possible therapeutic targets.

Extensive deposition of collagens and other ECM proteins represent biomarkers for activated fibroblasts of SSc. SPARC and CTGF are two such ECM proteins important

in regulating the production of collagen. TGF β is a multifunctional growth factor and cytokine and serves as a well-known inducer of ECM components, including collagen, SPARC and CTGF. On the other hand, SPARC and CTGF can regulate the TGF β signaling transduction leading to the alteration of collagen and other ECM protein production (19-23). Several mechanisms of such regulation have been explored, such as through SPARC and/or CTGF directly down-stream regulation or feedback control of TGF β signaling transduction, and through direct binding to TGF β receptor or TGF β itself (19-24). In our previous study, it was shown that SPARC siRNA can attenuate the production of some ECM components, such as type 1 and 3 collagens, SPARC and CTGF in human normal and SSc fibroblasts (16,17,24). We and others also showed that the blockade of CTGF expression either by CTGF siRNA, or its antisense oligonucleotide or corresponding antibody can block TGF β induced collagen production and/or fibronectin expression (24-27). Therefore, both SPARC and CTGF appear to have the potential to serve as a therapeutic target for fibrotic diseases such as SSc.

When the transcript levels of *Col1a2*, *Sparg*, *Ctgf* and *Tgfb1* were compared among the wild type and *Ctgf* transgenic mice fibroblasts, it was interesting to found that although the *Ctgf* expression in homozygote is higher than that in heterozygote, the expression of *Col1a2* in homozygote is surprisingly lower than that in heterozygote. The *Tgfb1* expression in homozygote is even not higher than that in wild type. When they were compared at the translational level, Col1 and *Ctgf* had lower expression in homozygote than that in heterozygote. It is somewhat similar to CTGF behavior in mink lung epithelial Mv1Lu cells that the activity of 3TP-luciferase, a TGF β reporter gene, can be induced by low CTGF concentration, such as 3 nM and 15 nM, while the stimulated level turned to decrease when the CTGF concentration increased to 35 nM (23). Both TGF β and Smad responsive elements have been identified in the CTGF promoter (28,29). Excessive amount of *Ctgf* might stimulate the secretion of TGF β and thus activate the TGF β pathway

in *Ctgf* transgenic heterozygous fibroblasts. However, too high expression of *Ctgf* in an early stage might cause negative feedback regulation to the TGF β pathway, which results in the down-regulation of TGF β and its down-stream genes in homozygous fibroblasts.

Similar to human fibroblasts, *Sparc* siRNA and *Ctgf* siRNA down-regulated the expression of collagen in mice fibroblasts, both wild type and *Ctgf* transgenic types at transcription and translation levels (Table1, Figures 3 and 4). The expression of the other two ECM proteins examined here, *Sparc* and *Ctgf*, also decreased. Therefore, the fibrosis in *Ctgf* transgenic mice model might be ameliorated by *Sparc* siRNA or *Ctgf* siRNA treatment. Furthermore, diminished level of TGF β were found in both *Sparc* siRNA and *Ctgf* siRNA treated fibroblasts, indicating that decreased *Sparc* and *Ctgf* can regulate the TGF β signaling pathway through directly reducing the TGF β expression level. The relationship between *Sparc* and TGF β /collagen in *Ctgf* transgenic mice fibroblasts also gave support of the previous observation that *Sparc* can magnify TGF β expression in cultured mouse mesangial cells, and that *Sparc*-null mesangial cells showed significantly decreased synthesis of TGF β and collagen (20,21). In mouse mesangial cells, lack of *Sparc* can result in the disturbance of Smad2 phosphorylation, which is synergistically induced by TGF β and endogenous *Sparc*. It was proposed that in mouse mesangial cells *Sparc* regulates the production of collagen type 1 via a TGF β dependent pathway through an interaction with TGF β -receptor complex in the presence of TGF β (20). In addition, a reciprocal, positive autocrine feedback loop between *Sparc* and TGF β might also explain the regulation of *Sparc* on TGF β expression and/or activation (21). Therefore, *Sparc* siRNA could modulate the composition of the ECM in *Ctgf* transgenic mouse fibroblasts via its effects on the levels of *Sparc* itself and TGF β .

Although, mRNA expression levels of *Sparc* and *Ctgf* showed a reciprocal inhibition of these two genes by corresponding siRNA treatment in mouse fibroblasts, translational levels of *Sparc* and *Ctgf* are partially discordant and concordant respectively,

to their mRNA levels. Sparc siRNA down-regulated Ctgf protein efficiently in all three type of fibroblasts (16%, 32%, and 40% in wide type, heterozygous and homozygous fibroblasts, respectively), and which was comparable with the effect of Ctgf siRNA on the Ctgf protein expression. However, Ctgf siRNA down-regulated the Sparc protein expression only in Ctgf homozygous fibroblasts (33%), but not in wild type and Ctgf heterozygous fibroblasts. Discordant expression levels of mRNA and protein of Sparc in both wide type and heterozygous fibroblasts treated with Ctgf siRNA may reflect negative feedback on the mRNA or the protein or the presence of different regulatory mechanisms that are not understood currently. On the other hand, concordant regulation of mRNA and protein of Ctgf by Sparc siRNA supports our previous finding in human fibroblasts, in which SPARC showed as an upstream regulator of CTGF in response to TGF β stimulation (24).

In conclusion, inhibition of Sparc or Ctgf expression by their corresponding siRNA in cultured fibroblasts of Ctgf transgenic mice showed a down-regulation of the expression of TGF β and collagen type I. Sparc and Ctgf siRNAs also showed a reciprocal inhibition in transcript levels, but Sparc siRNA functioned more efficiently than Ctgf siRNA in reducing the protein level of the Sparc and Ctgf. The present *in vitro* study using fibroblasts from Ctgf transgenic mouse models provided useful and potentially sufficient information for *in vivo* research to proceed.

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Table 1. Inhibition of gene expression by siRNA.

Fibroblast line	Gene name	Relative transcription level (mean \pm SD)		
		Non-Targeting siRNA	Ctgf siRNA	Sparc siRNA
Wide type mice	<i>Col1a2</i>	1	0.38 \pm 0.09	0.59 \pm 0.06
	<i>Ctgf</i>	1	0.27 \pm 0.16	0.74 \pm 0.07
	<i>Sparc</i>	1	0.71 \pm 0.22	0.31 \pm 0.06
	<i>Tgfb1</i>	1	0.40 \pm 0.05	0.50 \pm 0.00
Ctgf transgenic mice (Heterozygote)	<i>Col1a2</i>	1	0.26 \pm 0.14	0.42 \pm 0.03
	<i>Ctgf</i>	1	0.15 \pm 0.08	0.38 \pm 0.06
	<i>Sparc</i>	1	0.47 \pm 0.14	0.18 \pm 0.08
	<i>Tgfb1</i>	1	0.30 \pm 0.08	0.48 \pm 0.15
Ctgf transgenic mice (Homozygote)	<i>Col1a2</i>	1	0.30 \pm 0.09	0.50 \pm 0.26
	<i>Ctgf</i>	1	0.13 \pm 0.07	0.60 \pm 0.32
	<i>Sparc</i>	1	0.60 \pm 0.12	0.32 \pm 0.19
	<i>Tgfb1</i>	1	0.61 \pm 0.01	0.65 \pm 0.22

Figure 1. Comparison of gene expression among the wide type, heterozygous and homozygous *Ctgf* transgenic mice fibroblasts. The expression level of each gene in wild type fibroblasts was normalized to 1. Bars show the mean \pm SD results of analysis of 3 independent experiments performed in triplicate. * $P < 0.05$.

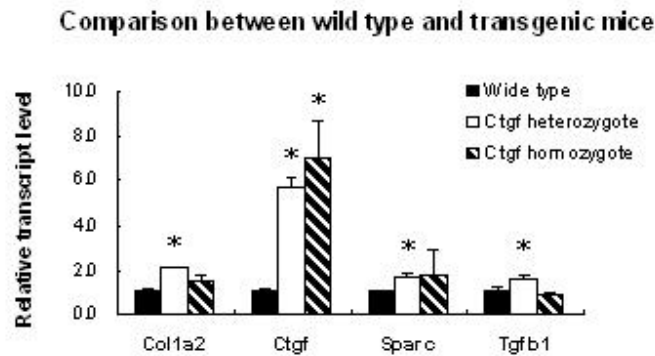


Figure 2. GFP expression in the fibroblasts from GFP transgenic mouse with or without GFP siRNA treatment. Left, without GFP siRNA treatment; right, with GFP siRNA treatment.

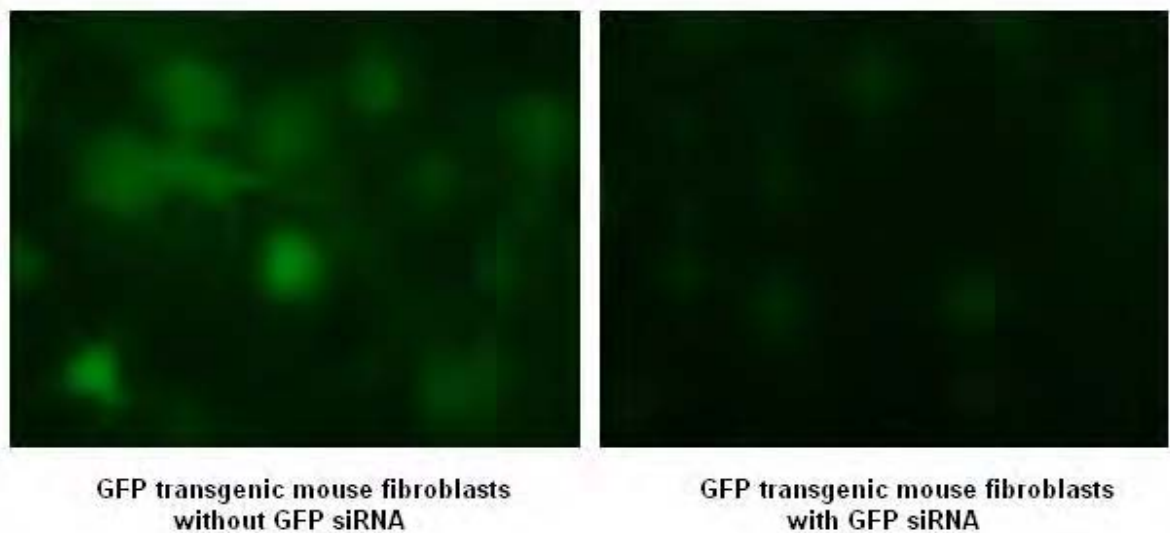


Figure 3. Gene expression in original and siRNA treated fibroblasts. **A, B, and C** are the comparison of gene expression with *Ctgf* siRNA, *Sparc* siRNA, or Non-Targeting siRNA treatment in wide type, heterozygous, and homozygous *Ctgf* transgenic mice fibroblasts, respectively. The expression level of each gene in each fibroblast line with Non-Targeting siRNA treatment was normalized to 1. * $P < 0.05$.

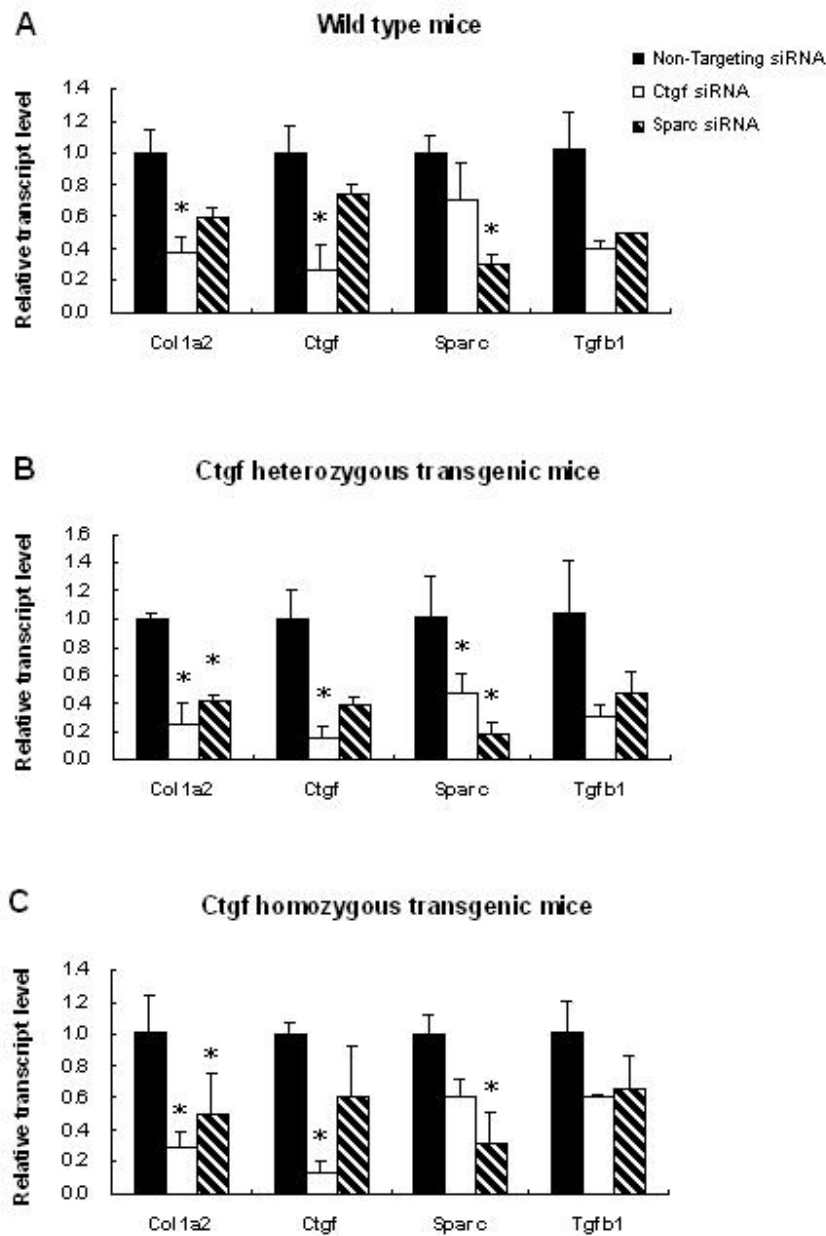
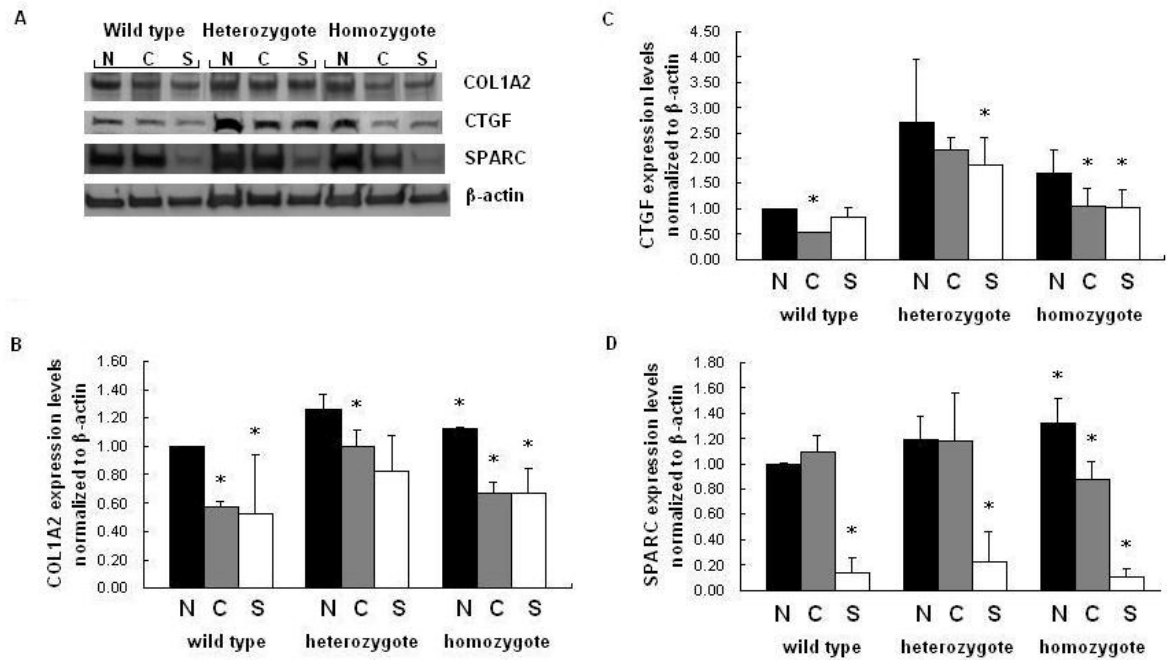


Figure 4. Western blot analysis of type I collagen, Ctgf, and Sparc in wild type, heterozygous, and homozygous Ctgf transgenic mice fibroblasts, with Ctgf siRNA or Sparc siRNA transfection (A). N = Non-Targeting siRNA treatment; C = Ctgf siRNA treatment; S = Sparc siRNA treatment. Wild type stands for wild type fibroblasts; Heterozygote stands for Ctgf transgenic mice fibroblasts heterozygote; Homozygote stands for Ctgf transgenic mice fibroblasts homozygote. Densitometric analysis of Western blots for Col1, Ctgf and Sparc are summarized in B, C, and D. Protein

expression levels were first compared between transgenic mice fibroblasts and wild type fibroblasts, and then between the same cell line with or without Ctgf/Sparc siRNA treatment. * $P < 0.05$.



Abstract 1

Association studies of genetic variants to the functions of human fibroblasts in response to environmental stimulation

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Objective: Fibroblasts play important roles in controlling fibrosis. Specific genetic variants, such as single nucleotide polymorphism (SNP), carried by individuals may associate with profibrotic activities of the fibroblasts. The purpose of this study was to identify genes with genetic variants associated with fibrotic changes in fibroblasts.

Methods: Thirty-four fibroblast strains were obtained from skin biopsies of different individuals. The fibroblasts were cultured and stimulated with silica particles with a time course and dose response. Gene expression of COL1A2, COL3A1, CTGF, SPARC, TIMP3 and TGFBR2 that are involved in activation of fibrosis were examined with real-time RT-PCR. Meanwhile, the fibroblast strains from different individuals were genotyped with Illumina's Human Tag SNP 317K panel. The associations between specific genotypes of the genes across the genome and the expressions of genes involved in activation of fibrosis were analyzed with linear model approach. The genes with SNPs associated with the expression patterns of the genes involved in activation of fibrosis were inhibited by siRNA transfection in cultured fibroblasts. Real-time quantitative RT-PCR and Western blots were used to examine transcript and/or protein levels of the genes involved in fibrosis. Assays were performed in triplicates. Marginal correlation and partial correlation methods were used to explore the gene-gene interaction in regulating the collagen expression.

Results: Specific genotypes of the CXCL12 were found to associate with gene expression of collagens (COL1A2 and COL3A1) and/or other genes (CTGF, SPARC, TIMP3 and TGFBR2) involved in activation of fibrosis. Silencing the CXCL12 gene with sequence specific siRNAs decreased the mRNA and/or protein expression ($p < 0.05$) of COL1A2 and COL3A1 in the fibroblasts. In addition, the expression changes in both the COL1A2 and the COL3A1 were found to be positively correlated with the gene expressions of SPARC, CTGF and TIMP3.

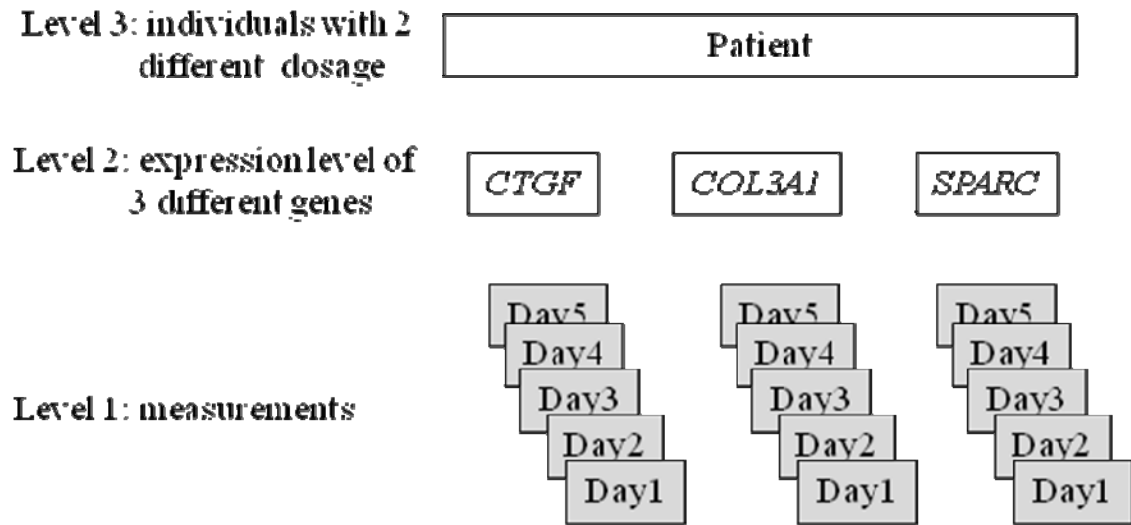
Conclusion: Inhibition of CXCL12 gene expression with sequence specific siRNAs in cultured human fibroblasts showed a down-regulation of the expression of both type I and type III collagen. Association studies of genetic variants to the functions of human fibroblasts in response to environmental stimulation may facilitate understanding of gene-gene interactions. Application of siRNA to silence CXCL12 may be a potential therapeutic approach to fibrotic disorders such as scleroderma.

Abstract 2

Single and multi-level multivariate longitudinal models in the genetic studies of time course gene expressions of human fibroblasts in response to environmental stimulation

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Single and multi-level multivariate longitudinal linear genetic models are developed for investigation of genetics of time-course gene expressions. Specifically, in the single longitudinal linear models expressions of a single gene is taken as a quantitative trait, which it is taken from the expression levels of three genes for multi-level multivariate longitudinal linear model. A locus influencing gene expression variation is referred to as eQTL. Time course gene expressions as responses to genetic and environmental perturbations are a function of time t . We can view the time-course gene expressions as a realization of a stochastic process and decompose them into mean function, and genetic additive effect and dominance effect functions using Fisher's factor analysis and variation method, which leads to a general genetic model of functional trait and formulates genetic effect functions as function of genotypic values and allele frequencies. The structure of multi-level multivariate longitudinal model is shown in Figure 1:



To evaluate the performance of the proposed models, we conduct pilot studies. We studied 79 fibroblast strains. We use RT-PCR to measure expressions of the genes COL1A2, COL3A1, CTGF, SPARC and TIMP3 in TGF β pathway with and without 10 μ g silica perturbation at 24-, 48-, 72-, 96- and 120-hour exposure of silica. All 79 fibroblast strains were genotyped by Illumina 317K SNP array. After checking the quality of typed SNPs, total of 313,932 SNPs were left for analysis. Single and multivariate longitudinal linear models each identified multiple genotypes (SNPs) associated with the expressions of collagen genes and three ECM genes, respectively. The most striking result is that both models identified that the SNP rs10484710 of the COL21A1 was strongly associated with the single gene expressions of COL1A2 and COL3A1, and with the combination expressions of COL3A1, CTGF and SPARC (Table 1).

Table 1. Associations between SNP rs10484710 and the expressions of the ECM genes

Single longitudinal Linear model		
gene expression	p value for regulating expression	OR
COL1A2	5.9×10^{-13}	1.4
COL3A1	1.65×10^{-5}	1.4
Multi-level multivariate longitudinal linear model		
gene expression	p value for regulating expression	OR
COL3A1, CTGF, SPARC	5.11×10^{-8}	1.4